

Rec'd PCT/PTO 2 5 JAN 2005
PCT/AU03/00951



**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

REC'D 14 AUG 2003

WIPO

PCT

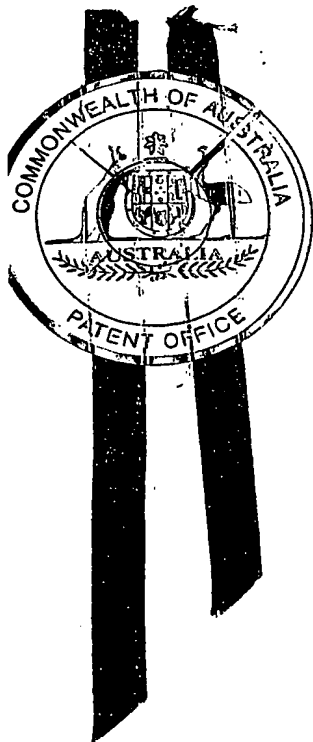
Patent Office
Canberra

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002950473 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION as filed on 26 July 2002.

WITNESS my hand this
Fifth day of August 2003

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES



BEST AVAILABLE COPY

141327236

AUSTRALIA

Patents Act 1990

Commonwealth Scientific and Industrial Research Organisation

PROVISIONAL SPECIFICATION

Invention Title:

Expression System

The invention is described in the following statement:

141327236

EXPRESSION SYSTEM

The present invention relates to a modified expression system for the transport and secretion of polypeptides. More specifically it relates to expression of proteins which readily fold in the cytoplasm.

BACKGROUND OF THE INVENTION

There is increasing interest in expression systems which enable bacterial production of recombinant proteins. In particular there is a need for systems which provide for high yield of recombinant proteins in culture supernatants particularly for agricultural and industrial uses. Recently, secretion of high yields of recombinant protein have been obtained using *Brevibacillus*.

In bacteria, following expression of a protein, transmembrane translocation of the protein can proceed by a number of routes depending on the nature of both the targeting signal and the substrate. In general, most proteins destined for export are synthesised with N-terminal extensions, called signal peptides. Signal peptides consist of short stretches of amino acids which, after protein delivery to the subcellular fraction, are removed. In bacteria two secretion pathways have been identified. The best characterised system is the Sec system for the general secretion of proteins. In this transport system, proteins are threaded through the membrane and correct folding occurs after transport.

The second system recently identified by Berks (1996) is the TAT system (for twin-arginine transfer peptide-dependent protein translocase). This system is so-called because the signal peptide generally contains the distinctive (S/T)RRXFLK motif. Proteins transported by this system are folded prior to translocation. Therefore, proteins of this type are toxic to bacteria if attempts are made to transport them through the membrane with a Sec secretion signal, significantly limiting the production and recovery of the enzyme in these systems.

In general, cytoplasmic proteins readily fold in the cytoplasm of heterologous hosts. Therefore it would be predicted that this group of proteins may not be readily secreted by the Sec system. β -galactosidase is a classic example of this. This particular enzyme from *E. coli* is a large protein with 120 kDa subunits and known to not pass through the cytoplasmic membrane when attached to a Sec-type secretion peptide

141327236

(Manoil & Beckwith, 1985; Bassford et al., 1979). An alternative system is required to facilitate recovery of proteins that readily fold in the cytoplasm.

One example of recombinant proteins which are required in high yield are enzymes which can be used to degrade organic pollutants.

5 Microorganisms are involved in the degradation of many organic compounds and are the principal agents for the biodegradation and recycling of organic matter. The degradation of organic compounds by microorganisms is primarily due to the action of various enzymes produced by the microorganism. The role of these enzymes in degrading organic pollutants has been investigated and a number of enzymes derived
10 from microorganisms have been identified that may be useful in assisting in bioremediation and the clean up of environmental pollutants and toxic compounds such as organophosphate pesticides.

Residues of organophosphate insecticides are undesirable contaminants of the environment and a range of commodities. Areas of particular sensitivity include
15 contamination of soil, irrigation tailwater that is re-cycled, used by irrigators downstream or simply allowed to run off-farm, and residues above permissible levels in meat and horticultural exports. Poisoning with organophosphates presents a problem for agricultural workers that are exposed to these chemicals, as well as military personnel exposed to organophosphates used in chemical warfare.
20 Furthermore, the stockpiling of organophosphorus nerve agents has resulted in the need to detoxify these stocks. Bioremediation strategies are therefore required for eliminating or reducing these organophosphate residues and/or stockpiles.

One proposed strategy involves the use of enzymes capable of immobilising or degrading the organophosphate residues. Such enzymes may be employed, for
25 example, in bioreactors through which contaminated water could be passed, or in washing solutions after post-harvest disinfestation of fruit, vegetables or animal products to reduce residue levels and withholding times. Suitable enzymes for degrading organophosphate residues include OP hydrolases from bacteria (Mulbry, 1992; Mulbry and Kearney, 1991; Cheng et al., 1999; US 5,484,728; US 5,589,386; Horne
30 et al., 2002; PCT/AU02/00594), vertebrates (Wang *et al.*, 1993; 1998; Gan *et al.*, 1991; Broomfield *et al.*, 1999) and OP resistant insects (PCT/AU95/00016 and PCT/AU96/00746).

The most thoroughly studied OP degrading enzyme is bacterial organophosphate dehydrolase (OPD), which is encoded by identical genes on
35 dissimilar plasmids in both *Flavobacterium* sp. ATCC 27551 and *Brevundimonas*

141327236

diminuta MG (Harper *et al.*, 1988; Mulbry and Karns, 1989). OPD is a homodimeric protein that is capable of hydrolysing a wide range of phosphate triesters (both oxon and thion OPs) with impressive kinetics (Dumas *et al.*, 1989a, b). Its reaction mechanism directly or indirectly involves metal ions, preferably Zn^{++} . OPD has no detectable activity with phosphate monoesters or diesters (Dumas *et al.*, 1989a, b; 1990).

OPD homologues (phosphotriesterase homology proteins, or PHPs) have been identified in the genomes of *Escherichia coli* (ePHP), *Mycobacterium tuberculosis* (mtPHP) and *Mycoplasma pneumoniae* (mpPHP), although only ePHP has been tested for phosphotriesterase activity (Scanlan and Reid, 1995; Buchbinder *et al.*, 1998). OPD homologues have also been identified in vertebrates (Davies *et al.*, 1997), although their function in these organisms is unknown.

OPD/OpdA have been identified as important enzymes that may assist in the bioremediation and clean up of environmental organophosphorus pollutants.

Microorganisms such as bacteria should provide an important production tool for OPD/OpdA and various systems have been employed to increase the production and output of this enzyme. However expression systems are not available to provide sufficient quantities of this enzyme to meet the growing demand.

The present inventors have developed a novel expression system comprising a modified signal peptide. This expression system has enabled the production of OpdA and other cytoplasmic proteins in high yields from *Brevibacillus*.

Summary of the Invention

In a first aspect the present invention provides a method of producing a heterologous polypeptide from a host cell comprising a TAT translocation system, the method comprising:

transforming the host cell with a DNA sequence encoding the heterologous polypeptide and a signal peptide wherein the signal peptide comprises a TAT signal, a Sec avoidance signal and a signal peptidase cleavage site;

culturing the host cell under conditions which allow expression of the heterologous polypeptide; and

recovering the heterologous polypeptide secreted from the host cell via the TAT translocation system.

Preferably, the host cell is *Bacillus sp.* More preferably, the host cell is *Bacillus choshinensis*, *Bacillus brevis*, *Bacillus subtilis*, *Bacillus licheniformis*, or

141327236

Bacillus megatorium. It is most preferred that the host cell is *Brevibacillus sp*, particularly *Bacillus choshinensis*.

In a further preferred embodiment the heterologous polypeptide is a polypeptide which readily folds in the cytoplasm. In particular embodiments the polypeptide is OpdA.

Preferably the mature heterologous polypeptide expressed by the method of the first aspect comprises the sequence provided in SEQ ID No:1 from residue 29 to 384; or a polypeptide which is greater than 90% identical to the sequence provided in SEQ ID No:1. More preferably, the polypeptide is at least 95% identical to the sequence provided in SEQ ID No:1, even more preferably at least 97% identical, and even more preferably at least 99% identical to the sequence provided in SEQ ID No:1.

While it is believed that the concept of signal sequences including a TAT signal are well known for the sake of clarity a number of such sequences are set out in Table 1.

Table 1 – A list of TAT sequences cited in Berks (1996). The signature "twin arginine" motif is in bold type.

MEARMTGRRKV TRRD AMADAARAVGVACLGGFSLAALVRTASPVDA
MSRSAKPQNG RRR FLRDVVRTAGGLAAVGVALGLQQQTARA
MTWS RRQ FLTGVGVLAAVSGTAGRVVA
MD RRR FLTLGSGAGLTATVATAGTAKA
MSEKDKMIT RRD ALRNIAVVVGSVATTTMMGVGVADA
MQIVNL TRG FLKAACVVTGGALISIRMTGKAVA
MNNEETFYQAM RRQ GVTRRSFLKYCSLAATSLGLGAGMAPKIAWA
MSTGTTNLVRTLDSMD FLKMD RRTFMKA VSALGATAFLGTYQTEIVNA
MKCYIGRGKNQVEERLERRGV SRRD FMKFCTAVAVAMGMGPAFAPKVAEA
MN RRN FIKAASCGALLTGALPSVSHA
MSHADEHAGDHGAT RRD FLYYATAGAGTVAAGAAWTLVNQMNP
MTQISGSPDVPDLG RRQ FMNLLTFGTITGVAAGALYPAVKYLIP

141327236

Table 1 – A list of TAT sequences cited in Berks (1996). The signature “twin arginine” motif is in bold type.

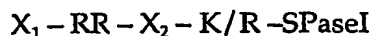
MDRRTFLRLYLLVGAAIAVAPVIKPALDYVGY
MTKLSGQELHAEL SR RAFLSYTAAVGALGLCGTSLLAQGARA
MTL TR REFIKHSGIAAGALVVTSAAPLPAWA
MTIS RR DLLKAQAAGIAAMAANIPLSSQAPA
MSEALSGRGND RR KFLKMSALAGVAGVSQAVG
MKTKIPDAVLAAEV SR RGLVKTТАIGGLAMASSALTLPFSRIAHA
MSNFNQIS RR DFVKASSAGAALAVSNLTLPFNVMA
MSIS RR SFLQGVGIGCSACALGAFPPGALA
MKTVLPSPETVRL SR RGFLVQAGTITCSVAFGSVPA
MGRLNRFRLGKDGR RE QASLSRRGFLVTSLGAGVMFGFARPSSA
MSDKDSKNTPQVPEKLGL SR RGFLGASAVTGAAVAATALGGAVMTRESWA
MESRT SR TFVKGLAAAGVLGGLGLWRSPSWA
MSLS RR QFIQASGIALCAGAVPLKASA
MTLNRRDFIKTSGAAVAAGILGFPHLAFG
MTDSRANRADATRGVASV RRR FLAGAGLTAGAIALSSMSTSASA

In a second aspect the present invention provides a signal peptide, the signal peptide having the sequence MKKRRVNSVLLLLLLASALALTVPMAKA.

5 In a third aspect the present invention provides a recombinant polynucleotide, the polynucleotide comprising a first and a second sequence, the first sequence encoding a signal peptide comprising a TAT signal, a Sec avoidance signal and a signal peptidase cleavage site and the second sequence encoding a heterologous protein.

141327236

In a preferred embodiment the sequence of the signal peptide is



in which X_1 is a sequence of 2 to 23 amino acids and X_2 is a sequence of 15 to 24 amino acids.

5 In another preferred embodiment the sequence of the signal peptide is MKKRRVNSVLLLLLLASALALTVPMAKA.

As will be understood by those skilled in this area the method of the present invention is suitable for expression of a number of cytoplasmic proteins, including but not limited to, lipases, proteases, esterases and enzymes involved in carbohydrate metabolism.

10 In a preferred embodiment the heterologous peptide is not *B. subtilis* Lipase A.

In a fourth aspect, the present invention provides a substantially purified polypeptide produced according to the method of the first aspect.

15 In another preferred embodiment the polynucleotide encoding the mature polypeptide has a sequence selected from:

- (i) a sequence of nucleotides shown in SEQ ID NO:2 from nucleotide 85 to 1155;
- (ii) a sequence encoding a polypeptide according to the second aspect; or
- 20 (iii) a sequence which is greater than 90% identical to SEQ ID NO:2 from nucleotide 85 to 1155.

The present invention also provides a suitable vector for the replication and/or expression of the polynucleotide. The vectors may be, for example, a plasmid or phage vector provided with an origin of replication, and preferably a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vector may contain one or more selectable markers, for example an ampicillin resistance gene in the case of a bacterial plasmid. The vector may be used *in vitro*, for example to transfect or transform a host cell.

25 In a further aspect, the present invention relates to the transformed host cell of the first aspect.

30 In a preferred embodiment the host cell is a *Brevibacillus sp.*, preferably *Bacillus choshinensis*. Such cells can be used for the production of commercially useful quantities of the encoded polypeptide.

141327236

In yet another aspect, the present invention provides a fusion protein comprising a polypeptide according to the first aspect fused to at least one other polypeptide sequence.

5 In a preferred embodiment of this aspect, the at least one other polypeptide is selected from the group consisting of: a polypeptide that enhances the stability of the polypeptide of the first or the second aspect, and a polypeptide that assists in the purification of the fusion protein.

In another aspect, the present invention provides a polynucleotide encoding the fusion protein.

10 The present invention also provides a composition for hydrolysing an organophosphate molecule, the composition comprising a polypeptide produced by the method of the first aspect of the present invention and one or more acceptable carriers.

15 In a still further aspect, the present invention provides a composition for hydrolysing an organophosphate molecule, the composition comprising the host cell of the present invention and one or more acceptable carriers.

20 It will be appreciated that in preferred embodiments the polypeptide can be used to hydrolyse organophosphates in a sample. For instance, after a crop has been sprayed with an organophosphate pesticide, the organophosphate residue can be hydrolysed from seeds, fruits and vegetables before human consumption. Similarly, organophosphate contaminated soil or water can be treated with a polypeptide of the second aspect of the present invention.

Detailed description of the invention

25 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

35 Organophosphates are synthetic organophosphorus esters and related compounds such as phosphoramidates. They have the general formula (RR'X)P=O or

141327236

(RR'X)P=S, where R and R' are short-chain groups. For insecticidal organophosphates X is a good leaving group, which is a requirement for the irreversible inhibition of acetylcholinesterase. The polypeptides of the present invention hydrolyse the phosphoester bonds of organophosphates.

5 Although well known for their use as pesticides, organophosphates have also been used as nerve gases against mammals. Accordingly, it is envisaged that the polypeptide of the present invention will also be useful for hydrolysis of organophosphates which are not pesticides.

10 By "substantially purified" we mean a polypeptide that has been separated from the lipids, nucleic acids, other polypeptides, and other contaminating molecules with which it is associated in its native state.

15 The % identity of a polypeptide is determined by FASTA (Pearson and Lipman, 1988) analysis (GCG program) using the default settings and a query sequence of at least 50 amino acids in length, and whereby the FASTA analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the FASTA analysis aligns the two sequences over a region of 100 amino acids.

20 Amino acid sequence mutants of the polypeptides of the present invention can be prepared by introducing appropriate nucleotide changes into a nucleic acid sequence, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics.

25 OPDA activity as used herein refers to the ability of the enzyme to hydrolase a organophosphate molecule. OPDA activity may be determined by, for example, assaying culture supernatant containing secreted OPDA for activity against an organophosphate molecule. The organophosphate molecule may be selected from the group consisting of: coumaphos, coroxon, paraoxon, parathion, parathion-methyl, phosmet, fenthion, diazinon, chlorpyrifos, and dMUP. More preferably, the
30 organophosphate is phosmet or fenthion.

 OPDA activity, such as activity against coumaphos, in for example, supernatant, may be compared to that in the cell fraction, or to the supernatant fraction obtained from a cell expressing a control, such as an unmodified OPDA.

35 Secretion of folded proteins via the Sec secretion system may be avoided by the inclusion of a Sec avoidance signal as shown in a study by Tjalsma *et al.* (2000)

141327236

examining the signal peptides of *Bacillus subtilis*. Tjalsma *et al.* examined both the Sec system and the TAT system. They found that a positively-charged residue (such as lysine or arginine) near the cleavage site acts as a Sec avoidance signal. Accordingly a Sec avoidance signal has been included in the OpdA polypeptide of the present invention to avoid transport of the polypeptide via the Sec secretory pathway and the subsequent toxic effect this transport would have on the cell. Furthermore, the secretion of the polypeptide from the host cell eliminates the need for cell disruption and protein refolding resulting in a high yield of purified polypeptide.

Signal peptidases remove signal peptides from secretory proteins. Signal peptidases such as signal peptidase I (SPase I) cleave the signal peptide at specific sites such as cleaving between contiguous alanine residues.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence preferably is active and capable of being transported via the TAT transport pathway, preferably having at least 25 to 50% of the activity of unmodified OpdA.

In general, preferably less than 20%, 10% or 5% of the amino acid residues of a variant or derivative are altered as compared with the corresponding region depicted in the sequence listing.

B. Polynucleotides and vectors.

Polynucleotides of the invention comprise nucleic acid sequences encoding the polypeptides of the invention. Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code.

141327236.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *Brevibacillus*.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Such vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

The vectors may be, for example, plasmid vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid. Vectors may be used *in vitro*, for example to transfect or transform a host cell.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, prokaryotic promoters may be used, in particular those suitable for use in *Brevibacillus* strains (such as *Bacillus brevis* and *Bacillus choshinensis*). A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as

141327236

a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in *E. coli* (Raibaud et al., Ann. Rev. Genet., 18: 173, 1984). In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter (U.S. Pat. No. 4,551,433). For example, the tac promoter is a hybrid trp-lac promoter comprised of both trp promoter and lac operon sequences that is regulated by the lac repressor (Amann et al., Gene, 25: 167, 1983; de Boer et al., Proc. Natl. Acad. Sci. USA, 80: 21, 1983). Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes.

Phage promoters may also be used, for example lambda. These promoters are readily available in the art.

Oligonucleotides and/or polynucleotides of the present invention may selectively hybridise to the sequence set out in SEQ ID NO:2 under high stringency. As used herein, stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄ at 50°C; (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42°C in 0.2 x SSC and 0.1% SDS.

141327236

C. Host cells

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/ polynucleotides and/or expressing the polypeptides of the invention encoded by the polynucleotides of the invention.

5 Suitable host cells include prokaryotes such as *Brevibacillus*.

Suitable host cells to transform include any cell that can be transformed with a polynucleotide of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule (e.g., nucleic acid molecules encoding one or more proteins of the present invention). Host
10 cells of the present invention can be capable of producing such proteins after being transformed with at least one nucleic acid molecule of the present invention. Host cells of the present invention can be any cell capable of producing at least one protein of the present invention.

Vectors/ polynucleotides of the invention may be introduced into suitable
15 host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. More preferred host cells are selected from the *Brevibacillus* cluster comprising the following species, namely, *Bacillus brevis*, *Bacillus agri*, *Bacillus centrosporus*, *Bacillus choshinensis*, *Bacillus parabrevis*, *Bacillus reuszeri*, *Bacillus formosus*, *Bacillus borstelensis*, *Bacillus laterosporus*, and *Bacillus thermoruber*. Even more preferred host cells are *Bacillus brevis* and *Bacillus choshinensis*.
20

Host cells of the present invention can be cultured in conventional fermentation bioreactors. The host cells can be cultured by any fermentation process which includes, but is not limited to, batch, fed-batch, cell recycle, and continuous
25 fermentation. Preferably, host cells of the present invention are grown by batch or fed-batch fermentation processes.

D. Protein Expression and Purification

Another embodiment of the present invention includes a recombinant cell
30 comprising a host cell transformed with one or more recombinant molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion.
35 Transformed nucleic acid molecules of the present invention can remain

141327236

extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Host cells comprising polynucleotides of the invention may be used to
5 express polypeptides of the invention. Host cells may be cultured under suitable conditions which allow expression of the polypeptide produced according to the invention. Expression of the polypeptides of the invention may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated
10 when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

Polypeptides of the invention can be collected from the supernatant of cultures of host cells expressing OpdA of the invention.

Purification of polypeptides may optionally be performed using well known
15 techniques such as affinity chromatography, including immunoaffinity chromatography, ion-exchange chromatography and the like, or affinity chromatography systems based on fusion protein sequences such as those known in the art.

Recombinant DNA technologies can be used to improve expression of
20 transformed polypeptide molecules by manipulating, for example, the number of copies of the polypeptide molecules within a host cell, the efficiency with which those polypeptide molecules are transcribed and the efficiency with which the resultant transcripts are translated. Recombinant techniques useful for increasing the expression of polypeptide molecules of the present invention include, but are not
25 limited to, operatively linking polypeptide molecules to high-copy number plasmids, integration of the polypeptide molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-
30 Dalgarno sequences), modification of polypeptide molecules of the present invention to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing polypeptide molecules encoding such a protein.

141327236

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described
5 in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

10 Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in Australia in the field relevant to the present invention

15 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

20 In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described by reference to the following non-limiting Examples.

141327236

Examples and resultsExpression of OpdA

5 The amino acid sequence of OpdA including the native signal peptide is shown in SEQ ID NO. 1. In this sequence the signal peptide is residues 1 to 28 and the sequence is set out below. Examination of this sequence shows that it possesses the distinctive twin arginine motif of the TAT system which implies that OpdA is folded prior to secretion and cleavage of the signal peptide. Therefore it is unlikely that

10 OpdA can be secreted through the Sec system.

MQTRRDALKSAAAITLLGGLAGCASMAR

(The signal peptide of OpdA with the TAT motif underlined.)

15 In an effort to obtain higher yields of OpdA expression of the protein was attempted using a Brevibacillus expression system developed by Higeta Shoya Co. Ltd. In using this system the native signal peptide was deleted and replaced with the following sequence:

MKKRRVNSVLLLLLLASALALTVPMAFAIAGS

20 *(I indicates SpaseI cleavage site)*

Attempts using this expression system were unsuccessful.

25 In an effort to understand this failure it was noted that while the modified signal peptide possessed the "twin arginine" motif of the TAT system it lacked a positively charged residue near the cleavage site which Tjalsma *et al.* (2000) had found acts as a Sec avoidance signal in *Bacillus subtilis*. In an effort to determine whether expression could be achieved by incorporation of a Sec avoidance signal, the signal peptide was modified to include a positively-charged residue and a "Bacillus" type signal peptidase (SPaseI) cleavage site.

141327236

Two complimentary oligos (NCMO25',
 5'GTTTCAGCCCATGGCTAAAGCTGCAGAGCACGGATCCGATC and NCMO23',
 5'GATCGGATCCGTGCTCTGCAGCTTTAGCCATGGGCTGAAC) containing an *Nco*I
 site and one end (underlined) and a *Bam*HI site at the other end (bold-type) were
 5 designed to alter the signal peptide to that shown below.

(a) MKKRRVVNSVLLLLLLASALALTVPMAFA | AGS

(b) MKKRRVVNSVLLLLLLASALALTVPMAKA | AEH

- 10 The amino acid sequence of the original signal peptide in pNCMO2 (a) and the altered
 signal peptide in pNC(mod) (b).

The two oligos were dissolved in TE buffer to 44 nmol/ml. Ten microlitres of
 each were incubated for 10 minutes at 95°C and then cooled slowly to room
 15 temperature. This would melt the two complimentary bands together. This was then
 digested with *Nco*I and *Bam*HI. The plasmid pNCMO2 was digested with *Nco*I and
*Bam*HI and extracted from a 1% agarose gel using the QIAquick PCR purification kit
 (QIAGEN). The digested pNCMO2 and digested oligos were ligated overnight and
 transformed the following day into *E. coli* DH10β. All transformations in *E. coli* were
 20 performed according to the revised Hanahan method (Sambrook *et al.*, 1989).
 Transformants were selected on LB agar plates (10 g/l tryptone, 5 g/l Yeast Extract,
 2.5 g/l NaCl) containing 100 µg/ml ampicillin. One transformant containing an
 altered signal peptide (as determined by DNA sequencing) was selected and
 designated pNC(mod).

25

Construction of pNC(mod)-*opdA*

The plasmid pNC(mod) was digested with *Bam*HI and *Eco*RI and excised
 from a 1% agarose gel using the QIAquick PCR purification kit (QIAGEN). The
 plasmid pGAfull was digested with *Bam*HI and *Eco*RI and the 1 kb *opdA* containing
 30 fragment was excised from a 1% agarose gel using the QIAquick PCR purification kit
 and ligated overnight with the extracted pNC(mod) fragment. The ligation mixture
 was transformed into *E. coli* CC118 (*araD*139, Δ(*ara*, *leu*)7697, Δ*lacX*74, *phoA*Δ20, *galE*,
galK, *thi*, *rpsE*, *rpoB*, *argE*_{am1}, *recA*1; Manoil & Beckwith, 1985) and transformants
 selected on LB plates containing ampicillin (100 µg/ml). The *opdA* expression in
 35 pNC(mod)-*opdA* is run from a *lacZ* promoter and there is leaky expression from *lac*

141327236

promoters in some *E. coli* strains. However, no expression from *lac* promoters occurs in *E. coli* CC118 due to deletion of the *lac* operon. Transformation into *E. coli* DH10 β resulted in point mutations in *opdA*, resulting in the production of a non-functional protein. This is probably due to an inability of the signal peptide to target the TAT secretion system and avoid the Sec system in *E. coli*.

Expression of *opdA* in *Brevibacillus choshinensis*

The plasmid pNC(mod)-*opdA* was transformed into *Brevibacillus choshinensis* HPD31 (Takagi *et al.*, 1989) using the Tris-PEG method of Udaoka & Yamagata (1993) and transformants selected on BTY medium (glucose, 1.0%; tryptone, 2.0%; Yeast Extract, 0.5%; FeSO₄·7H₂O, 0.001%; MnSO₄·4H₂O, 0.001%; ZnSO₄·7H₂O, 0.001%) with 20 mM MgCl₂ and 50 μ g/ml neomycin at 28°C. One transformant was picked and grown up for further analysis. The plasmid contained in this transformant was extracted and examined and shown to be pNC(mod)-*opdA*. *B. choshinensis* pNC(mod)-*opdA* was grown to mid-log phase and induced with 1 mM IPTG at 28°C for 24 hours in BTY medium with 50 μ g/ml neomycin. The culture supernatant and cells were separated by centrifugation (7000 *g*, 10 minutes). The cell pellet was resuspended in 2 ml 50 mM Tris-HCl pH7.5 and sonicated.

Tris-PEG method of transformation

B. choshinensis was streaked onto BTY medium and grown at 37°C for 2 days. A loopful of growth was then used to inoculate 5 ml of BTY medium and grown overnight at 37°C. The overnight culture was diluted 100-fold in 5 ml of the same medium and incubated at 37°C for 5 hours. The cells were then pelleted by centrifugation (4000 *g*, 5 minutes) and washed with 5 ml of 50 mM Tris-HCl pH7.5. The pellet was finally resuspended in 5 ml of 50 mM Tris-HCl pH8.5 and incubated for 30 minutes at 37°C. After this incubation the cell pellet was washed with 1 ml of MTP. MTP was prepared as follows:

0.1 M Sodium Maleate pH6.5	20 ml
Phosphate Buffer(7% w/v K ₂ HPO ₄ / 2.5% w/v KH ₂ PO ₄)	10 ml
H ₂ O	18 ml
Autoclave and then add:	
1 M MgCl ₂ (filter-sterilised)	2 ml
BTY	50 ml

141327236

Plasmid DNA was added to the cell suspension, after which 1.5 ml of a PEG solution (40 g PEG8000, 20 ml 0.1 M Sodium maleate pH6.5 and H₂O to 100 ml) was added and the mixture incubated at room temperature for 2 minutes. MTP (5 ml) was added and mixed well. The cells were collected by centrifugation (4000 *g*, 10 minutes at room temperature). The cells were then resuspended in 1 ml of BTY with 20 mM MgCl₂ and incubated at 30°C for 2.5 hours with moderate shaking.

Checking for expression

Both the supernatant and the cell extracts were examined for coumaphos hydrolytic activity and SDS-PAGE. No coumaphos hydrolytic activity could be detected in *B. choshinensis* cells in the absence of pNC(mod)-opdA. The majority of the coumaphos hydrolytic activity was secreted into the culture supernatant (Table 2). The specific activity of the supernatant was 5.09 $\mu\text{mol}/\text{min}/\text{mg}$ protein), which is close to that of purified OpdA (8.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein), suggesting that OpdA in the supernatant is relatively pure.

Table 2 – The percentage coumaphos hydrolytic activity in *B. choshinensis* with various plasmids.

Strain	Supernatant	Cells
<i>B. choshinensis</i> pNC(mod)-opdA	64.8 \pm 7.6	35.2 \pm 4.2
<i>B. choshinensis</i> pNC(mod)	nd ¹	nd

nd¹ = not detected

The signal peptide allows secretion of active cytoplasmic proteins

In general, cytoplasmic proteins readily fold in the cytoplasm of heterologous hosts. Therefore it would be predicted that this group of proteins may not be readily secreted by the Sec system. β -galactosidase is a classic example of this. This particular enzyme from *E. coli* is a large protein of 120 kDa and known to not pass through the cytoplasmic membrane when attached to a Sec-type secretion peptide (Manoil & Beckwith, 1985; Bassford *et al.*, 1979). Therefore, this protein was chosen to examine the secretion of active cytoplasmic proteins by this *Brevibacillus* system.

141327236

The *lacZ* gene was amplified by PCR using pEM32m as a template. The primers used were lac5c (5'CATGTCGACATGGATCCCGTCGTT) and lac3b (5'CATGAATTCTTATTTTGAAGTGGTAA) containing *Sa*I and *Eco*RI sites, respectively. The PCR was performed using the *Pfu* Turbo DNA polymerase from Stratagene, according to the manufacturer's instructions. The 3 kb PCR product was purified using the QIAquick PCR purification kit and ligated with pGEM T Easy (Promega) overnight. The ligation mixture was then transformed into *E. coli* DH10 β and transformants selected on LB plates containing ampicillin (100 μ g/ml), X-Gal (5-bromo-4-chloro-3-indolyl β -D galactoside, 40 μ g/ml) and IPTG (40 μ g/ml). It was noted that there were some intense blue colonies on the plates. One was examined and shown to contain the 3 kb PCR product encoding β -galactosidase. This plasmid was called pGEMlac and demonstrates the presence of a functional β -galactosidase enzyme. When this insert was contained in the opposite orientation, no β -galactosidase activity was generated (Table 3).

Table 3 – The β -galactosidase activity in *E. coli* DH10 β cells with various constructs.

Strain	β -galactosidase activity (nmol/min/ μ g protein)
<i>E. coli</i> DH10 β pGEMlac2 ¹	0.0054 \pm 0.0009
<i>E. coli</i> DH10 β pGEMlac5 ²	11.001 \pm 0.007

¹*lacZ* is contained in the opposite orientation to *lac* promoter

²*lacZ* is contained in the same orientation as the *lac* promoter

The *Sa*I-*Eco*RI fragment containing β -galactosidase was ligated with similarly-digested pNC(mod). The ligation mix was transformed into *E. coli* CC118 and transformants selected on LB plates containing ampicillin (100 μ g/ml). Several transformants were picked and examined for inserts. One was chosen and shown to contain the 3 kb *Sa*I-*Eco*RI fragment. This plasmid was designated pNC(mod)-lac. This plasmid was then transformed into *Brevibacillus* and transformants selected on BTY medium with 50 μ g/ml neomycin at 30°C. One colony was picked after two days growth and examined for the production of β -galactosidase when induced with IPTG. The culture was pelleted by centrifugation (7000g, 10 minutes) and the cell pellet resuspended in 50 mM Tris-HCl pH7.5, after which it was sonicated. Both the cell extract and the culture supernatant were examined for β -galactosidase activity. The

141327236

majority of the -galactosidase activity was contained in the supernatant (Table 4). *Brevibacillus choshinensis* does not possess any intrinsic β -galactosidase activity.

Table 4 – The percentage β -galactosidase activity in *B. choshinensis* cells with various plasmids.

Strain	Supernatant	Cells
<i>B. choshinensis</i> pNC(mod)-lac	98.2 \pm 6.0	1.80 \pm 0.17
<i>B. choshinensis</i> pNC(mod)	nd ¹	nd

¹not detected

This secretion system was tested on another cytoplasmic protein, that is also a phosphotriesterase. Horne *et al.* (2002b) identified the protein HocA from a *Pseudomonas monteilli* strain that is capable of hydrolysing organophosphates. This protein is a 20 kDa cytoplasmic protein unrelated in sequence and reaction mechanism to OpdA. The *hocA* gene was amplified by PCR using the upstream and downstream oligonucleotide primers, hoc5,

5'GTCTAAGGATCCATGAAAGAAGAACTAAAAACC, and hoc3, 5'GTCTAAAAGCITTTACCAGTTTAGCTTTAG, with *Bam*HI and *Hind*III restriction sites (underlined), respectively, and the template pBSRK7(1) as a template. The PCR product was digested with *Bam*HI and *Hind*III and cloned into similarly-digested pK18. The ligation was transformed into *E. coli* DH10 β . One transformant was chosen and shown by sequence analysis to have a correct *hocA* gene. This clone was designated pKhoc2. The 501 bp *hocA*-containing *Bam*HI-*Hind*III fragment of pKhoc2 was then cloned into pNC(mod) and transformed into *E. coli* CC118. One clone was selected that possessed *hocA* and this clone was designated pNC(mod)-hoc. The plasmid pNC(mod)-hoc was transformed into *Brevibacillus choshinensis* with transformants selcted on BTY medium with 50 μ g/ml neomycin at 30°C. One colony was picked after two days growth and grown in 50 ml BTY medium to mid-log phase and then induced with 1 mM IPTG at 28°C for 24 hours. The culture supernatant and cells were separated by centrifugation (7000*g*, 10 minutes). The cell pellet was resuspended in 50 mM Tris-HCl pH7.5 and sonicated. The coumaphos hydrolytic activity in the cell extract and in the culture supernatant was determined. Table 5

141327236

shows the relative amounts of activity. The majority of the protein was secreted in an active form into the culture supernatant.

5 Table 5 – The percentage coumaphos hydrolytic activity in *B. choshinensis* with various plasmids

Strain	Supernatant	Cells
<i>B. choshinensis</i> pNC(mod)	nd ¹	nd
<i>B. choshinensis</i> pNC(mod)-hoc	75.1±6.8	24.9±0.9

¹not detected

10 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this day of 2002

Commonwealth Scientific and
Industrial Research Organisation
Patent Attorneys for the Applicant:

BLAKE DAWSON WALDRON
PATENT SERVICES

141327236

References

- Bassford, Jr., P.J., T.J. Silhavy & J.R. Beckwith. 1985.** Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. *J. Bacteriol.* **139**: 19-31.
- 5 **Berks, B.C. 1996.** A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* **22**: 393-404.
- Harcourt, R.L., I. Horne, T.D. Sutherland, B.D. Hammock, R.J. Russell & J.G. Oakeshott. 2002.** Development of a simple and sensitive fluorimetric method for the isolation of coumaphos-hydrolysing bacteria from the environment. *Lett. Appl. Microbiol.* **34**: 263-268.
- 10 **Horne, I, T.D. Sutherland, R.L. Harcourt, R.J. Russell & J.G. Oakeshott. 2002a.** Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl. Environ. Microbiol.* **68**: 3371-3376
- 15 **Horne, I., T.D. Sutherland, J.G. Oakeshott & R. J. Russell. 2002b.** Cloning and expression of the phosphotriesterase gene, *hoca*, from *Pseudomonas montelli* C11. *Microbiol.* Accepted for publication.
- 20 **Machowski, E.E., R.A. McAdam, K.M. Derbyshire & V. Mizrahi. 2000.** Construction and application of mycobacterial reporter transposons. *Gene* **253**: 67-75.
- Manoil, C. & J. Beckwith. 1985.** *Tnp_{hoA}*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* **82**: 8129-8133.
- 25 **Miyauchi, A., M. Ozawa, M. Mizukami, K. Yashiro, S. Ebisu, T. Tojo, T. Fujii & H. Takagi. 1999.** Structural conversion from non-native to native form of recombinant human epidermal growth factor by *Brevibacillus choshinensis*. *Biosci. Biotechnol. Biochem.* **63**: 1965-1969.
- 30 **Morris, S.H. & C.C. Adley. 2001.** Irish public perceptions and attitudes to modern biotechnology: an overview with a focus on GM foods. *Trends in Biotechnol.* **19**: 43-48.

141327236

Nagarajan, V. 1990. System for secretion of heterologous proteins in *Bacillus subtilis*. *Methods Enzymol.* 185: 214-228.

5 Neu, H.C. & L.A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of sphaeroplasts. *J. Biol. Chem.* 240: 3685-3692.

10 Rowland, S.S., J.J. Zulty, M. Sathyamoorthy, B.M. Pogell & M.K. Speedie. 1992. The effect of signal sequences on the efficiency of secretion of a heterologous phosphotriesterase by *Streptomyces lividans*. *Appl. Microbiol. Biotechnol.* 38: 94-100.

Sambrook, J., E.F. Fritsch & T. Maniatis. 1989. Molecular cloning – A Laboratory Manual. 2nd Ed. Cold Spring Harbour Laboratory Press, USA.

15 Simon, R., U. Priefer & A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technol.* 1: 784-791.

20 Steiert, J.G., B.M. Pogell, M.K. Speedie & J. Laredo. 1989. A gene coding for a membrane-bound hydrolase is expressed as a secreted soluble enzyme in *Streptomyces lividans*. *Bio/Technol.* 7: 65-68.

25 Takagi, H., K. Kadowski & S. Udaka. 1989. Screening and characterization of protein hyper-producing bacteria without detectable exoprotease activity. *Agric. Biol. Chem.* 53: 691-699.

Tjalsma, H., A. Bolhuis, J.D. Jongbloed, S. Bron & J.M. van Dijk. 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* 64: 515-547.

30 Udaka, S. & H. Yamagata. 1993. High-level secretion of heterologous proteins by *Bacillus brevis*. *Meth. Enzymol.* 217: 23-33.

141327236

SEQUENCE LISTING

<110> Commonwealth Scientific and Industrial Research Organisation

5 <120> Expression System

<160> 10

<170> PatentIn Ver. 2.1

10

<210> 1

<211> 384

<212> PRT

<213> Agrobacterium radiobacter

15

<400> 1

Met Gln Thr Arg Arg Asp Ala Leu Lys Ser Ala Ala Ala Ile Thr Leu
1 5 10 15

20

Leu Gly Gly Leu Ala Gly Cys Ala Ser Met Ala Arg Pro Ile Gly Thr
20 25 30Gly Asp Leu Ile Asn Thr Val Arg Gly Pro Ile Pro Val Ser Glu Ala
35 40 45

25

Gly Phe Thr Leu Thr His Glu His Ile Cys Gly Ser Ser Ala Gly Phe
50 55 60

30

Leu Arg Ala Trp Pro Glu Phe Phe Gly Ser Arg Lys Ala Leu Ala Glu
65 70 75 80Lys Ala Val Arg Gly Leu Arg His Ala Arg Ser Ala Gly Val Gln Thr
85 90 95

35

Ile Val Asp Val Ser Thr Phe Asp Ile Gly Arg Asp Val Arg Leu Leu
100 105 110Ala Glu Val Ser Arg Ala Ala Asp Val His Ile Val Ala Ala Thr Gly
115 120 125

40

Leu Trp Phe Asp Pro Pro Leu Ser Met Arg Met Arg Ser Val Glu Glu
130 135 140

45

Leu Thr Gln Phe Phe Leu Arg Glu Ile Gln His Gly Ile Glu Asp Thr
145 150 155 160Gly Ile Arg Ala Gly Ile Ile Lys Val Ala Thr Thr Gly Lys Ala Thr
165 170 175

50

Pro Phe Gln Glu Leu Val Leu Lys Ala Ala Ala Arg Ala Ser Leu Ala
180 185 190Thr Gly Val Pro Val Thr Thr His Thr Ser Ala Ser Gln Arg Asp Gly
195 200 205

55

Glu Gln Gln Ala Ala Ile Phe Glu Ser Glu Gly Leu Ser Pro Ser Arg
210 215 220

60

Val Cys Ile Gly His Ser Asp Asp Thr Asp Asp Leu Ser Tyr Leu Thr
225 230 235 240Gly Leu Ala Ala Arg Gly Tyr Leu Val Gly Leu Asp Arg Met Pro Tyr
245 250 255

141327236

Ser Ala Ile Gly Leu Glu Gly Asn Ala Ser Ala Leu Ala Leu Phe Gly
 260 265 270
 5 Thr Arg Ser Trp Gln Thr Arg Ala Leu Leu Ile Lys Ala Leu Ile Asp
 275 280 285
 Arg Gly Tyr Lys Asp Arg Ile Leu Val Ser His Asp Trp Leu Phe Gly
 290 295 300
 10 Phe Ser Ser Tyr Val Thr Asn Ile Met Asp Val Met Asp Arg Ile Asn
 305 310 315 320
 Pro Asp Gly Met Ala Phe Val Pro Leu Arg Val Ile Pro Phe Leu Arg
 325 330 335
 15 Glu Lys Gly Val Pro Pro Glu Thr Leu Ala Gly Val Thr Val Ala Asn
 340 345 350
 20 Pro Ala Arg Phe Leu Ser Pro Thr Val Arg Ala Val Val Thr Arg Ser
 355 360 365
 Glu Thr Ser Arg Pro Ala Ala Pro Ile Pro Arg Gln Asp Thr Glu Arg
 370 375 380
 25
 30 <210> 2
 <211> 1155
 <212> DNA
 <213> Agrobacterium radiobacter
 35 <400> 2
 atgcaaacga gaagagatgc acttaagtct gcggccgcaa taactctgct cggcggttg 60
 gctgggtgtg caagcatggc ccgaccaatc ggtacaggcg atctgattaa tactgttcgc 120
 ggccccattc cagtttcgga agcgggcttc acactgacct atgagcatat ctgcggcagt 180
 40 tcggcggggat tcctacgtgc gtggccggag tttttcggta gccgcaaagc tctagcggaa 240
 aaggctgtga gaggattacg ccatgccaga tcggctggcg tgcaaaccat cgtcgatgtg 300
 tcgactttcg atatcggtcg tgacgtccgt ttattggccg aagtttcgcg ggccgcccgc 360
 gtgcataatcg tggcggcgac tggcttatgg ttcgaccgcg cactttcaat gcgaatgcgc 420
 agcgtcgaag aactgaccca gttcttcctg cgtgaaatcc aacatggcat cgaagacacc 480
 45 ggtattaggg cgggcattat caaggtcgcg accacaggga aggcgacccc ctttcaagag 540
 ttggtgttaa aggcagccgc gcgggccagc ttggccaccg gtgttcgggt aaccactcac 600
 acgtcagcaa gtcagcgaga tggcgagcag caggcagcca tatttgaatc cgaaggtttg 660
 agccccctcac gggtttgbat cggtcacagc gatgatactg acgatttgag ctacctaac 720
 ggcctcgctg cgcgcggata cctcgtcggg ttagatcgca tgccgtacag tgcgattggg 780
 ctagaaggca atgcgagtcg attagcgctc tttggtactc ggtcgtggca aacaagggt 840
 50 ctcttgatca aggcgtcat cgaccgagc tacaaggatc gaatcctcgt ctcccatgac 900
 tggctgttcg ggttttcgag ctatgtcacg aacatcatgg acgtaatgga tcgcataaac 960
 ccagatggaa tggccttcgt ccctctgaga gtgatcccat tcctacgaga gaagggcgtc 1020
 ccgcccgaaa cgctagcagg cgtaaccgtg gccaatcccc cgcggttctt gtcaccgacc 1080
 gtgcgggccg tcgtgacagc atctgaaact tcccgcctg ccgcgcctat tccccgtcaa 1140
 55 gataaccgaac gatga 1155

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.